PAK is required for the disruption of E-cadherin adhesion by the small GTPase Rac

Encarnación Lozano[1,2,*], Marieke A. M. Frasa[1], Katarzyna Smolarczyk[1], Ulla G. Knaus[3] and Vania M. M. Braga[1,‡]

[1]Molecular Medicine Section, NHLI, Faculty of Medicine and [2]Ecology and Evolution Research Section, Faculty of Life Sciences, Imperial College London, London, SW7 2AZ, UK
[3]Department of Immunology, The Scripps Research Institute, La Jolla, CA 92037, USA

*Present address: Museo Nacional de Ciencias Naturales, CSIC, 28006 Madrid, Spain
‡Author for correspondence (e-mail: v.braga@imperial.ac.uk)

Accepted 14 January 2008
Journal of Cell Science 121, 933-938 Published by The Company of Biologists 2008
doi:10.1242/jcs.016121

Summary

E-cadherin cell-cell adhesion plays a major role in the maintenance of the morphology and function of epithelial tissues. Modulation of E-cadherin function is an important process in morphogenesis and tumour de-differentiation. We have previously shown that constitutively active Rac1 induces the disassembly of E-cadherin complexes from junctions in human keratinocytes. Here, we compare this activity in three members of the Rac subfamily (Rac1, Rac3 and Rac1b) and investigate the molecular mechanisms underlying Rac1-induced destabilization of junctions. We demonstrate that Rac3 shares with Rac1 the ability to interfere with cadherin-mediated adhesion. Rac1b is an alternative splice variant of Rac1 but, surprisingly, Rac1b cannot induce junction disassembly. Thus, Rac family members differ on their potential to perturb keratinocyte cell-cell contacts. The mechanism through which Rac promotes disassembly of cadherin-dependent adhesion does not involve an increase in contractility. Instead, activation of the Rac target PAK1 is necessary for destabilization of cell-cell contacts. Inhibition of PAK1 by dominant-negative constructs or depletion of endogenous PAK1 by RNA interference efficiently blocked Rac1-induced perturbation of junctions. Interestingly, PAK1 cannot be activated by Rac1b, suggesting that this may contribute to the inability of Rac1b to disrupt cell-cell contacts in keratinocytes. As PAK1 also plays a crucial role in lamellipodia formation, our data indicate that PAK1 is at the interface between junction destabilization and increased motility during morphogenetic events.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/7/933/DC1

Key words: Rac small GTPase, E-cadherin, PAK, Cell-cell adhesion, Keratinocytes

Introduction

E-cadherin is one of the most important cell-cell adhesion receptors involved in tissue morphogenesis and maintenance of epithelial tissue integrity (Gumbiner, 2005). Loss of function of E-cadherin has emerged as an important event for local invasion and metastasis (Christofori and Semb, 1999). Furthermore, E-cadherin expression becomes down-regulated when epithelial cells acquire motile and invasive characteristics (i.e. during epithelial wound healing and embryonic development) (Thiery, 2003).

The small GTPase Rac1 is an important modulator of the actin cytoskeleton and a key regulator of E-cadherin function (Gumbiner, 2005). Activation of Rac1 perturbs the distribution of E-cadherin at junctions in different cell types (Akhtar and Hotchin, 2001; Braga, 2000; Edme et al., 2002; Keely et al., 1997; Quinlan, 1999; Shintani et al., 2006; Yagi et al., 2007). This effect is specific because other surface receptors, such as integrins, are not removed from cell-cell contacts with the same time frame as cadherins (Braga et al., 2000).

In vivo, Rac1-dependent disruption of junctions has been shown during intestinal epithelia differentiation (Stappenbeck and Gordon, 2000), salivary gland morphogenesis (Pirraglia et al., 2006) and tracheal tubulogenesis (Chihara et al., 2003). Furthermore, Rac activation is also required to perturb E-cadherin junctions after Ras activation (Braga et al., 2000; Edme et al., 2002), Csk kinase expression (Yagi et al., 2007) and HGF/scatter factor treatment (Ridley et al., 1995; Takaishi et al., 1994). Yet, these effects may be cell-type specific (Braga et al., 1999; Lozano et al., 2003).

Upregulation of Rac1 protein and mRNA levels has been demonstrated in human tumours of epithelial origin, implicating Rac1 in cellular transformation (Lozano et al., 2003; Sahai and Marshall, 2002a). Two other family members, Rac3 and Rac1b, have also been found overexpressed or activated in several epithelial tumours. Rac3 is divergent from Rac1 at the C-terminal region (Haataja et al., 1997; Mira et al., 2000). Rac1b is an alternative splice variant of Rac1 (Jordan et al., 1999; Schnelzer et al., 2000) with an in-frame insertion of 19 amino acids in a region important for Rac1 interaction with regulators and effectors. Rac1b can neither induce lamellipodia nor activate the two known Rac1 effectors p21 activated kinase (PAK) and the downstream signaling pathway of Jun N-terminal kinase 1 (JNK1) (Fiegen et al., 2004; Matos et al., 2003). Yet, the functional consequences of Rac1, Rac3 and Rac1b activation are only now beginning to be unravelled (Chan et al., 2005; Matos et al., 2003). Furthermore, it remains to be tested whether Rac3 and Rac1b have similar functions as Rac1 on cell-cell adhesion.

The precise mechanisms by which E-cadherin stability at junctions is perturbed by Rac1 are still unclear. Here, we set out to

address whether activation of Rac3 or Rac1b can disrupt cadherin-dependent adhesion and identify specific effectors that can mediate this effect. We found that, in contrast to Rac1 and Rac3, Rac1b is not able to induce any substantial changes in E-cadherin cell-cell adhesion. This striking Rac1b phenotype indicates that differences in activation of signaling pathways by Rac1 and Rac1b may provide clues on the mechanism involved. Interestingly, PAK1 – a target that is not activated by Rac1b – is essential for Rac1 to perturb keratinocyte junctions. As PAK is also involved in epithelial morphogenesis and lamella formation (Sells et al., 1997; Tang et al., 1997), our results suggest that PAK1 is a pivotal Rac target that coordinates junction disassembly and increased motility.

Results and Discussion

We addressed whether, similarly to Rac1, Rac3 and Rac1b can also destabilize cadherins from keratinocyte cell-cell contacts. From four hours onwards after expression of the constitutively active Rac1 mutant $Rac1^{Q61L}$, E-cadherin localization at junctions was discontinuous with thickening of E-cadherin staining at the central region of cell-cell contacts. At later time points, cells partially detached from each other (Fig. 1a). A similar pattern and kinetics of junction disassembly was observed when constitutively active Rac3 ($Rac3^{G12V}$) was expressed and compared with $Rac1^{Q61L}$ (Fig. 1b). In contrast to Rac1 and Rac3, prolonged expression of constitutively active Rac1b ($Rac1b^{Q61L}$) or wild-type Rac1b (data not shown) did not result in any substantial alteration of E-cadherin distribution (Fig. 2a). Quantitatively, Rac1b-expressing cells showed high levels of intact junctions as opposed to Rac1 and Rac3 (Fig. 2b). These results are interesting, considering that Rac1b is identical to Rac1 apart from an insert of 19 amino acids (Jordan et al., 1999).

The inability of Rac1b to remove E-cadherin from junctions could be due to degradation of Rac1b protein. However, exogenous Rac1b protein was stable in keratinocytes. First, exogenous Rac1b was clearly expressed at levels comparable with those of activated Rac1 (Fig. 2c) when detected by an anti-Myc antibody (recognizes the Myc epitope in exogenous Rac1 and Rac1b) or an antibody against Rac1 (recognizing endogenous and exogenous Rac1 and Rac1b). Second, transfection experiments showed that Rac1b was not degraded (Fig. 2d). Although Rac1b mRNA is expressed in keratinocytes (data not shown), endogenous Rac1b was not detected consistent with its low levels of expression and high turnover.

Furthermore, Rac1 is the main Rac family member expressed in normal keratinocytes. Considering the transfection efficiency (around 30-40% in our hands), levels of exogenous Rac1 are about threefold higher than those of endogenous Rac levels (Fig. 2d), which is in the lower range of expression of exogenous proteins. Taken together with the lack of effect of overexpressed Rac1b, our data argue that disassembly of cadherin adhesion by Rac1 did not result from overexpression per se [for additional controls and specificity see Braga et al. (Braga et al., 2000)].

One of the mechanisms known to participate in the disruption of cell-cell contacts is increased contractility (Sahai and Marshall, 2002b; Zhong et al., 1997). Rac activation can also participate in contractile events by counterbalancing Rho activity and other mechanisms (Bokoch, 2003). However, inhibition of contractility was ineffective in preventing junction disruption by Rac1 (supplementary material Fig. S1). We reasoned that differences in the phenotypes observed between Rac1b and Rac1/Rac3 may be due to distinct signaling pathways that are activated by each GTPase. As Rac1b can not transduce signals through JNK1 and PAK1 activation (Fiegen et al., 2004; Matos et al., 2003; Singh et al., 2004), Rac1 and Rac3 could mediate junction disassembly through activation of one or both of these kinases. However, JNK1-dependent pathways were not necessary for junction breakdown induced by active Rac1 (supplementary material Fig. S2).

PAK is a serine/threonine kinase and one of the best characterized Rac targets: it participates in cell motility, cytoskeletal rearrangements and is able to phosphorylate many different cytoskeletal and cytosolic proteins (Bokoch, 2003). We inhibited PAK function using three different constructs: a kinase-dead version of PAK1 ($PAK1^{K299R}$) (Tang et al., 1997), a kinase-dead PAK1 that does not bind Rac ($PAK^{H83L,H86L,K299R}$) (Sells et al., 1997; Tang et al., 1997) and the auto-inhibitory domain of PAK1, which does not interact with active Rac (amino acid residues 83-149; PAK^{AID}). PAK^{AID} interacts in trans with the kinase domain of PAK1, PAK2 and PAK3, therefore blocking their ability to phosphorylate downstream targets (Zhao et al., 1998).

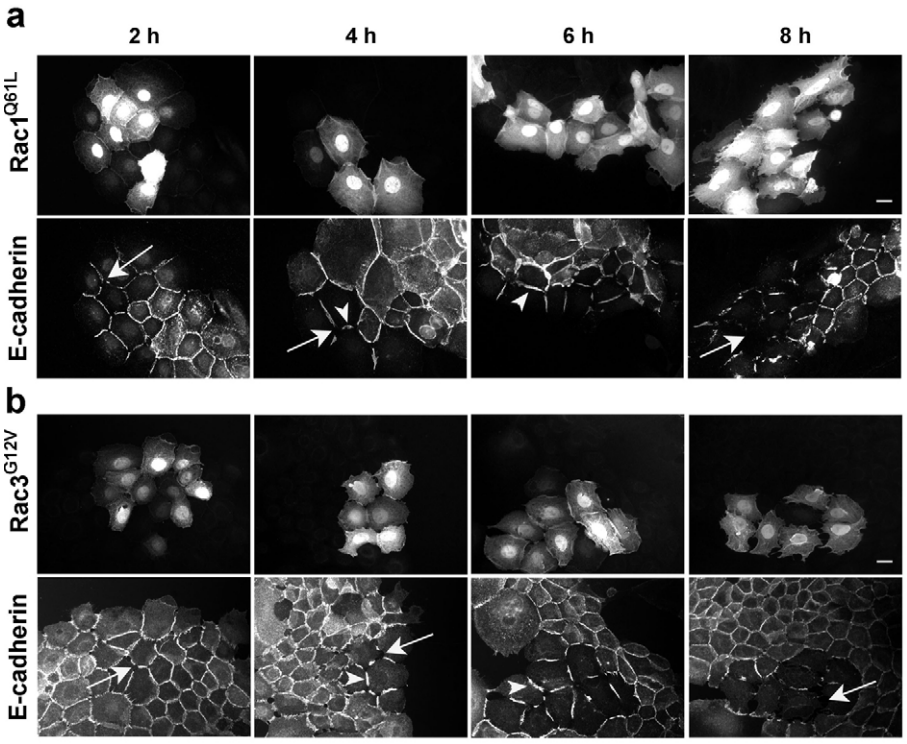

Fig. 1. Destabilization of E-cadherin–catenin complexes from keratinocyte junctions. Normal human keratinocytes were microinjected with (a) $Rac1^{Q61L}$ and (b) $Rac3^{G12V}$ cDNA, expressed for different time points (2-8 hours) and stained with anti-Myc and anti-E-cadherin antibodies. Arrows indicate absence of E-cadherin. Arrowheads show thickening of E-cadherin staining in the middle of cell-cell contact areas. Scale bars, 20 μm.

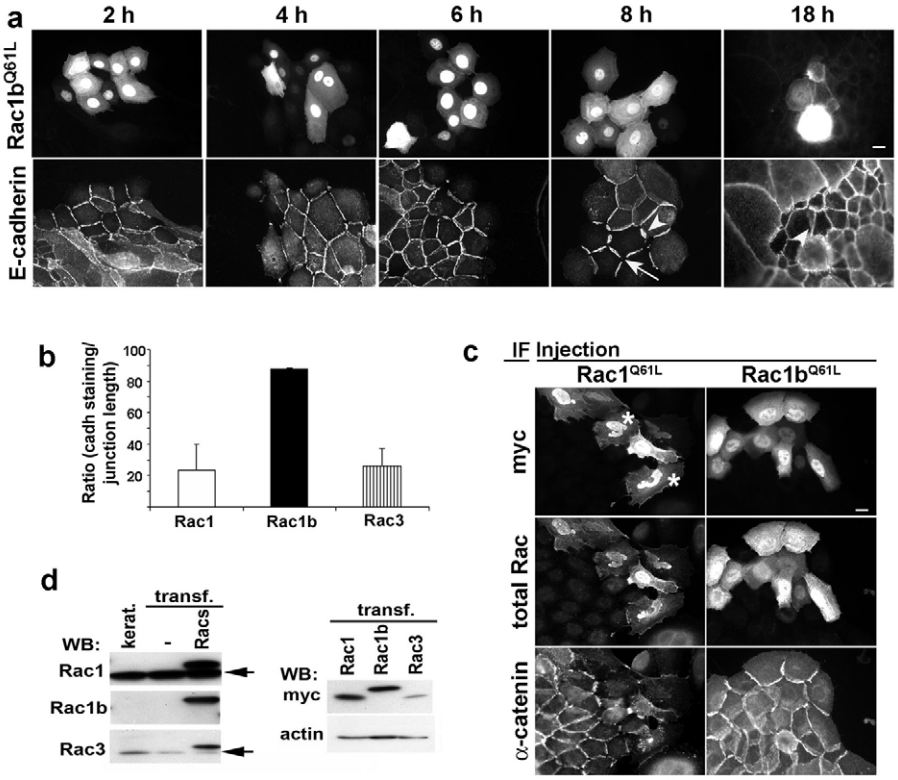

Fig. 2. Rac1b activation does not affect E-cadherin localization at junctions. (a) The constitutively active Rac1b mutant ($Rac1b^{Q61L}$) was expressed for up to 18 hours and stained with Myc-tagged and E-cadherin. Arrow indicates absence of E-cadherin staining; arrowheads show concentration of E-cadherin at cell-cell contact sites. (b) Quantification of the ratio of cadherin staining over junction length after an 8-hour expression of activated Rac1b, Rac1 and Rac3 (see Materials and Methods for details). Values are expressed as the percentage of the total number of junctions found in expressing cells. (c) Rac1b protein is not degraded after microinjection in keratinocytes. Active forms of Rac1 and Rac1b were expressed for 6 hours and detected using anti-Myc antibody (recognizes only exogenous Rac) or an anti-Rac monoclonal antibody [recognizes an epitope in both exogenous and endogenous Rac1 and Rac1b (total Rac)]. Junctions were visualized with α-catenin staining. Asterisks show cells with low expression of $Rac1^{Q61L}$ in which disruption of cell-cell contacts was achieved. (d) Keratinocytes were transfected with activated forms of Rac1, Rac1b and Rac3. Protein lysates were obtained and probed with antibodies against Rac1, Rac1b, Rac3, Myc and actin; arrows indicate endogenous Rac. Scale bars, 20 μm.

Inhibition of PAK alone did not disrupt junctions, indicating that PAK function per se is not essential for cadherin-dependent adhesion (Fig. 3a,b). Co-expression of active Rac1 with any of these inhibitory constructs effectively blocked junction disruption. By contrast, PAK inhibition was unable to rescue junction disassembly induced by active Arf6, suggesting the specificity of this effect for Rac1 (our unpublished data). Furthermore, it is unlikely that the effects observed by blocking PAK function resulted from unbalancing other Rac1-dependent pathways (Fig. 3b): (1) only PAK1 is expressed in keratinocytes (Fig. 3c) and, (2) inhibition of other signaling pathways triggered by Rac1 did not interfere with junction disassembly (supplementary material Figs S1 and S2, and data not shown).

To confirm that endogenous PAK is required downstream of Rac1 to disrupt cell-cell contacts, we performed RNA interference (RNAi) to deplete PAK1 using three different oligonucleotides (Fig. 4a-c). Optimization experiments showed that levels of PAK1 were effectively reduced (70-80%), but not E-cadherin protein levels (Fig. 4b). When constitutively active Rac1 was expressed in PAK1-siRNA-treated keratinocytes (Fig. 4a), cadherin staining along junctions was significantly restored (60%) when compared with control siRNA (25%, Fig. 4c). The rescue effect observed with PAK1 siRNA was smaller than when dominant-negative PAK constructs were used (90%, Fig. 3). The most possible explanation is the population-based quantification used in the RNAi experiments, in which all Rac1-expressing cells were considered. This criterion reduces the level of the effects quantified: some keratinocytes would not show rescue of junction disruption as they would still have some endogenous PAK. Alternatively, although unlikely, we cannot formally exclude the possibility that the three dominant-negative PAK constructs used inhibit additional pathways than the one leading to PAK activation. Nevertheless, our data indicate that endogenous PAK is necessary for disruption of cell-cell contacts induced by Rac1.

How can PAK1 participate in the Rac1-dependent downregulation of cadherin from junctions? We excluded the participation of lamella formation and increased contractility as the cause of junction disassembly induced by Rac (supplementary material Fig. S1) (Braga et al., 2000). An attractive possibility involves PAK1-mediated phosphorylation of proteins necessary for junction stability. Alternatively, PAK1 could potentially participate in the internalization of membranes containing E-cadherin receptors, because PAK has been involved in pinocytosis (Bokoch, 2003; Kumar and Vadlamudi, 2002). Indeed, our results suggest that E-cadherin partially colocalized with active Rac1 in intracellular vesicles (E.L., unpublished), in agreement with previous observations (Akhtar and Hotchin, 2001; Akhtar et al., 2000).

The potential mechanisms described above are not mutually exclusive, because PAK1-dependent phosphorylation of junction-associated proteins may signal to induce internalization (Gumbiner, 2005). A number of reports suggest the importance of cadherin internalization (Le et al., 1999; Lock and Stow, 2005) and phosphorylation of cadherin-associated proteins in cadherin turnover (Fujita et al., 2002; Gavard and Gutkind, 2006). Overall, whether the reduced presence of E-cadherin at keratinocyte junctions following Rac activation results from defective recycling and/or increased degradation is unclear at the moment and requires further work.

Rac1 has been shown to participate downstream of signaling pathways known to control cadherin stability at junctions in physiological or pathological conditions, i.e. downstream of growth factor receptors (Lozano et al., 2003; Sahai and Marshall, 2002a). In particular, stimulation of endothelial cells with VEGF (Gavard and Gutkind, 2006) or TNFα (Nwariaku et al., 2004) requires Rac and/or PAK signaling to promote internalization of VE-cadherin.

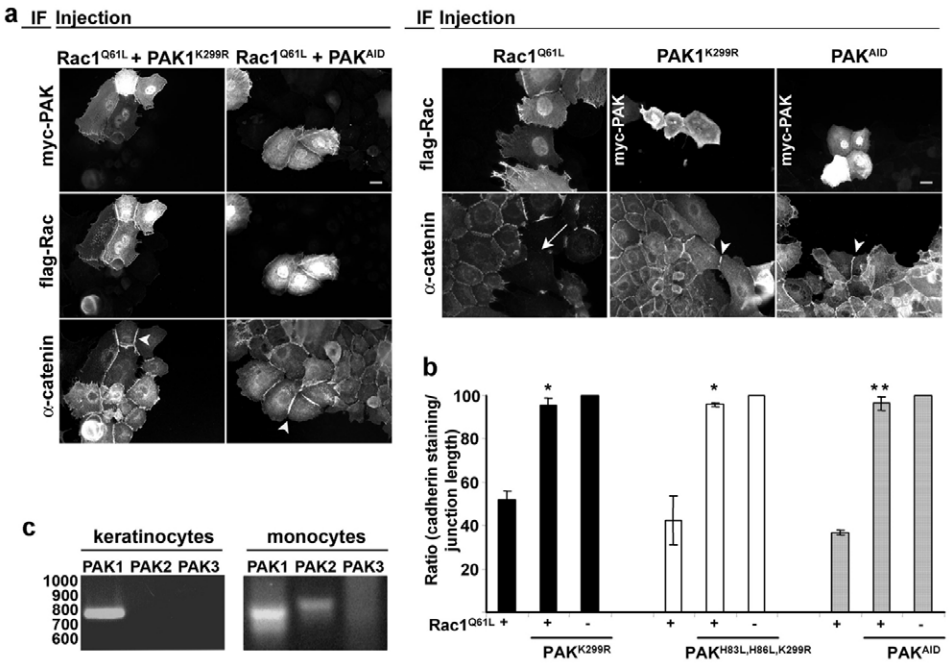

Fig. 3. PAK is necessary for Rac1-induced destabilization of E-cadherin complexes from junctions. (a, left panels) $Rac1^{Q61L}$ was co-expressed with kinase-dead $PAK1^{K299R}$ or with the auto-inhibitory domain Myc-PAK^{AID}. Seven hours post-injection, cells were fixed and immunostained for epitope tags and α-catenin. (Right panels) As controls, $Rac1^{Q61L}$, PAK^{K299R} or Myc-tagged PAK^{AID} were microinjected alone and stained as above. Arrow indicates absence of α-catenin. Arrowheads show the presence of α-catenin at cell-cell contacts. Scale bars, 20 μm. (b) Effects of co-expression of constitutively active Rac1 and PAK mutants were quantified by measuring the ratio of the length in cadherin staining to the length of the cell-cell contact (corner to corner of neighbouring expressing cells). Controls were arbitrarily set as 100% (length in cadherin staining = length of the cell-cell contact). *P<0.05; **P<0.01. (c) Keratinocytes express PAK1 mRNA, but not PAK2 or PAK3 mRNAs. Keratinocyte and monocyte RNAs were subjected to RT-PCR using primers specific for members of the class I PAK family.

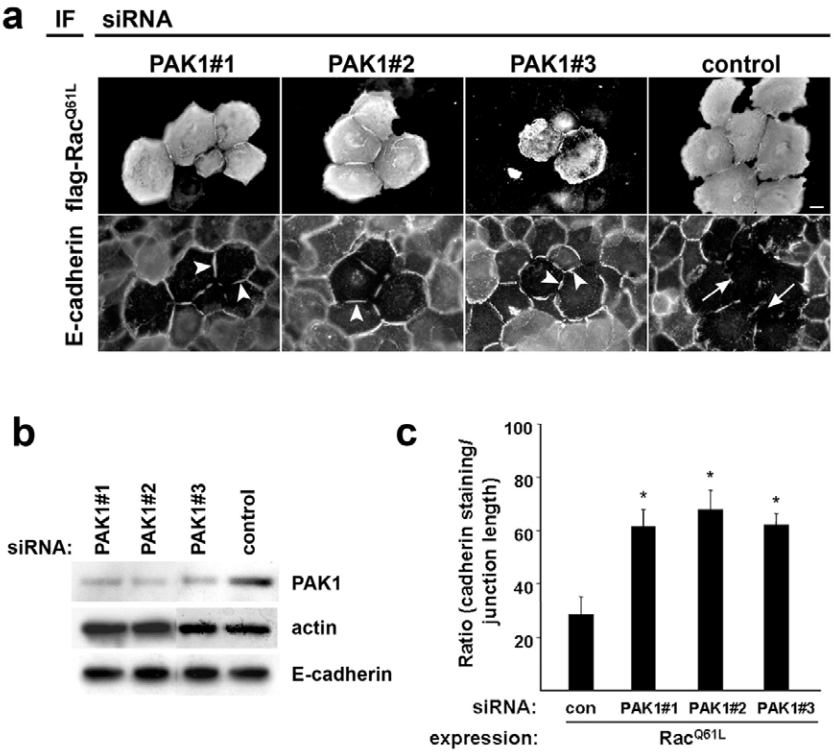

Fig. 4. (a) siRNA targeting PAK1 or control siRNA oligos were transfected into keratinocytes. Following expression of constitutively active Rac1 (0.5 mg/ml) for 7 hours, siRNA-treated keratinocytes were stained for E-cadherin and the FLAG-tag. Scale bar, 20 μm. (b) Lysates were obtained and probed with antibodies against PAK1, actin and E-cadherin. (c) The rescue of Rac-dependent junction disruption by depletion of endogenous PAK was quantified using the ratio between cadherin staining and junction length (see Materials and Methods). *P<0.05.

However, the fine mechanistic details downstream of PAK may vary according to cell type and stimuli (this work) (Braga et al., 1999; Gavard and Gutkind, 2006; Nwariaku et al., 2004). Taken together, these data point out that PAK1 might be a pivotal Rac target in the regulation of cadherin stability following different stimuli that destabilize junctions. As such, our work opens up exciting new avenues to investigate the mechanisms through which PAK influences adhesive and morphogenetic processes.

PAK has an emerging role in epithelial morphogenesis, differentiation and cell-cell contact inhibition of motility (Wang et al., 2003; Zegers et al., 2003). PAK1 function per se is not necessary for the maintenance of cadherin adhesion in keratinocytes (Figs 3, 4). Consistent with our results, constitutive activation of Mbt, the *Drosophila* homologue of PAK4, a class II PAK-family member, disrupts adherens junctions during eye development (Menzel et al., 2007). To our knowledge, we present the first evidence that PAK1 participates in the destabilization of E-cadherin following Rac1 activation. PAK1 activation correlates with transformation, increased invasion and hyperplasia (Kumar and Vadlamudi, 2002; Tang et al., 1997). Owing to its established role in lamellae protrusion (Bokoch, 2003), PAK might be an ideal Rac target to participate in junction disruption and, at the same time, stimulate migration in epithelial cells.

Materials and Methods

Cell culture

Normal human keratinocytes (strains Kb and Sf, passages 3-7) were cultured on a mitomycin-C-treated monolayer of 3T3 J2 fibroblasts at 37°C and 5% CO_2 as described (Rheinwald, 1989). For RNAi experiments, keratinocytes were seeded in normal medium, transferred to low-Ca^{2+} medium and grown until confluence (Braga et al., 1997).

Antibodies and reagents

Antibodies used were: anti-Myc epitope (9E10, CR-UK and JAC6, Serotec); anti-HA epitope (262K, Cell Signaling and SG77, Zymed); anti-E-cadherin (HECD1 and ECCD-2); anti-FLAG epitope (M2, Sigma); anti-Rac (23A8, Upstate); anti-Rac1b (gift from Peter Jordan, Centro de Genetica Humana, Portugal); anti-PAK (N20, Santa Cruz); anti-actin (C4, ICN Biomedicals) and anti-α-catenin (VB1) (Braga et al., 1995). Anti-Rac3 antibody was prepared in rabbits using amino acids 182-192 of human Rac3 as antigen. The affinity-purified antibody does not recognize purified or overexpressed Rac1 or Rac2. Secondary antibodies were from Jackson Immuno Research Laboratories. Y-27632, ROCK inhibitor, was a gift from A. Yoshimura, Yoshitomi Pharmaceuticals, Japan).

cDNA contructs

Full-length constitutively active $Rac1^{Q61L}$, $Rac1b^{Q61L}$ and $Rac3^{G12V}$ mutants were subcloned into the pCS2-myc vector. $Rac1^{Q61L}$ was subcloned into pRK5-myc and pRK5-FLAG. PAK auto-inhibitory domain (PAK^{AID}) and ROCKα kinase-dead in pRK5-myc were a gift from Alan Hall (Memorial Sloane-Kettering Cancer Center, New York, NY). $PAK1^{K299R}$ and $PAK1^{H83L,H86L,K299R}$ were provided by Jonathan Chernoff (Fox Chase Cancer Centre, Philadelphia, PA). JNK1(APF) was a gift of Roger Davis (University of Massachusetts Medical School, Boston, MA) and $CrkII^{R38K}$ and $CrkII^{W169K}$ mutants from Hidesaburo Hanafusa (Osaka Bioscience Institute, Osaka, Japan).

Microinjection

Unless specified, cDNA constructs were injected at 0.1 mg/ml into the nucleus of 50-100 cells per coverslip as described previously (Braga et al., 1997). For ROCK inhibition experiments, cells injected with activated Rac1 cDNA plasmid were incubated for 5 hours with 25 μM Y-27632. Microinjected keratinocytes were fixed, stained and analyzed as previously described (Braga et al., 1997).

RNA interference

Keratinocytes grown in low-Ca^{2+} medium were transfected with 50 nM of siRNA targeting PAK1 [5′-AAUCUGUAUACACACGGUCUG-3′, D-003521-07 and D-003521-01 (Dharmacon)] or siCONTROL non-targeting siRNA (Dharmacon) using RNAiFect (Qiagen)] according to manufacturer's instructions. Around 66 hours post transfection, cell-cell contacts were induced by addition of Ca^{2+} for 1 hour. Cells were microinjected with pRK5-FLAG-Rac^{Q61L} and incubated for 7 hours. PAK1 knockdown was confirmed by western blotting for each experiment.

Transfection

Keratinocytes grown in standard medium were transfected with 2 μg of plasmids encoding Myc-tagged, activated forms of Rac1, Rac1b and Rac3 and TransIT®-Keratinocyte Transfection Reagent (Cambridge Biosciences) according to the manufacturer's protocol. After overnight incubation cells were lysed in 50 mM Tris-HCl pH 7.5, 1% Triton X-100, 150 mM NaCl and protease inhibitors.

RT-PCR

RNA was extracted from keratinocytes and HNP-1 monocytes (gift from Emmanuele Caron, Imperial College London, UK) using RNAzolB (Biogenesis) and in Super-Script™ II reverse transcriptase reactions according to the manufacturer's protocol (Invitrogen). Specific primers were designed for PAK1 5′-ATACTGGATG-GCACCAGAGG-3′ and 5′-CTGGGCAGTTGAGTCACAGA-3′; PAK2 5′-AATT-GACCCTGTTCCTGCAC-3′ and 5′-CCCGAAATATTGGGGAAAGT-3′; PAK3 5′-CAAGGGGCATCAGGTACTGT-3′ and 5′-ATCAGAGGAGTCAGGCTGGA-3′. PCR products were resolved on 1% agarose gel and visualized under UV light.

Quantification

Only junctions between two expressing cells were considered for quantification, which was performed in different ways. First, to determine the disruption of junctions by Rac family members and rescue experiments, length measurements were performed for each junction (Leica LCS-Lite): the length of E-cadherin staining and the length of the cell-cell contact (corner to corner of neighbouring cells) for each contact. Their ratio was calculated; controls were set to 100% (i.e. cadherin staining = length of the cell-cell contact). In RNAi experiments, all Rac1-expressing cells were considered in the analysis becsause of the poor performance of anti-PAK1 antibodies regarding immunofluorescence. Statistical analysis was performed with Student's *t*-test; error bars give the standard error of the mean (± s.e.).

We thank E. Caron, J. Chernoff and A. G. Sáez for critical reading of the manuscript and all researchers listed therein for kindly providing reagents. We also thank J. Erasmus for technical assistance. E.L. was supported by the Ministerio de Ciencia y Tecnología, Spain, and Cancer Research UK; M.A.M.F. is a recipient of a PhD studentship from Association for International Cancer Research. V.M.M.B.'s lab is funded by the Medical Research Council and Cancer Research UK.

References

Akhtar, N. and Hotchin, N. A. (2001). RAC1 regulates adherens junctions through endocytosis of E-cadherin. *Mol. Biol. Cell* **12**, 847-862.

Akhtar, N., Hudson, K. R. and Hotchin, N. A. (2000). Co-localization of Rac1 and E-cadherin in human epidermal keratinocytes. *Cell Adhes. Commun.* **7**, 465-476.

Bokoch, G. M. (2003). Biology of the P21-activated kinases. *Annu. Rev. Biochem.* **72**, 743-781.

Braga, V. (2000). Epithelial cell shape: cadherins and small GTPases. *Exp. Cell Res.* **261**, 83-90.

Braga, V. M., Machesky, L. M., Hall, A. and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* **137**, 1421-1431.

Braga, V. M., Hodivala, K. J. and Watt, F. M. (1995). Calcium-induced changes in distribution and solubility of cadherins, integrins and their associated cytoplasmic proteins in human keratinocytes. *Cell Adhes. Commun.* **3**, 201-215.

Braga, V. M., Del Maschio, A., Machesky, L. and Dejana, E. (1999). Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. *Mol. Biol. Cell* **10**, 9-22.

Braga, V. M., Betson, M., Li, X. and Lamarche-Vane, N. (2000). Activation of the small GTPase Rac is sufficient to disrupt cadherin-dependent cell-cell adhesion in normal human keratinocytes. *Mol. Biol. Cell* **11**, 3703-3721.

Chan, A. Y., Coniglio, S. J., Chuang, Y.-y., Michaelson, D., Knaus, U. G., Philips, M. R. and Symons, M. (2005). Roles of the Rac1 and Rac3 GTPases in human tumor cell invasion. *Oncogene* **24**, 7821-7829.

Chihara, T., Kato, K., Taniguchi, M., Ng, J. and Hayashi, S. (2003). Rac promotes epithelial cell rearrangement during tracheal tubulogenesis in Drosophila. *Development* **130**, 1419-1428.

Christofori, G. and Semb, H. (1999). The role of the cell-adhesion molecule E-cadherin as a tumour-suppressor gene. *Trends Biochem. Sci.* **24**, 73-76.

Edme, N., Downward, J., Thiery, J.-P. and Boyer, B. (2002). Ras induces NBT-II epithelial cell scattering through the coordinate activities of Rac and MAPK pathways. *J. Cell Sci.* **115**, 2591-2601.

Fiegen, D., Haeusler, L. C., Blumenstein, L., Herbrand, U., Dvorsky, R., Vetter, I. R. and Ahmadian, M. R. (2004). Alternative splicing of Rac1 generates Rac1b, a self-activating GTPase. *J. Biol. Chem.* **279**, 4743-4749.

Fujita, Y., Krause, G., Scheffner, M., Zechner, D., Leddy, H. E., Behrens, J., Sommer, T. and Birchmeier, W. (2002). Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. *Nat. Cell Biol.* **4**, 222-231.

Gavard, J. and Gutkind, J. S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. *Nat. Cell Biol.* **8**, 1223-1234.

Gumbiner, B. M. (2005). Regulation of cadherin-mediated adhesion in morphogenesis. *Nat. Rev. Mol. Cell Biol.* **6**, 622-634.

Haataja, L., Groffen, J. and Heisterkamp, N. (1997). Characterization of RAC3, a novel member of the Rho family. *J. Biol. Chem.* **272**, 20384-20388.

Jordan, P., Brazao, R., Boavida, M. G., Gespach, C. and Chastre, E. (1999). Cloning of a novel Rac1b splice variant with increased expression in colorectal tumors. *Oncogene* **18**, 6835-6839.

Keely, P. J., Westwick, J. K., Whitehead, I. P., Der, C. J. and Parise, L. V. (1997). Cdc42 and Rac1 induce integrin-mediated cell motility and invasiveness through PI(3)K. *Nature* **390**, 632-636.

Kumar, R. and Vadlamudi, R. K. (2002). Emerging functions of p21-activated kinases in human cancer cells. *J. Cell. Physiol.* **193**, 133-144.

Le, T. L., Yap, A. S. and Stow, J. L. (1999). Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. *J. Cell Biol.* **146**, 219-232.

Lock, J. G. and Stow, J. L. (2005). Rab11 in recycling endosomes regulates the sorting and basolateral transport of E-cadherin. *Mol. Biol. Cell* **16**, 1744-1755.

Lozano, E., Betson, M. and Braga, V. M. M. (2003). Tumour progression: small GTPases and loss of cell-cell adhesion. *BioEssays* **25**, 452-463.

Matos, P., Collard, J. G. and Jordan, P. (2003). Tumor-related alternatively spliced Rac1b is not regulated by Rho-GDP dissociation inhibitors and exhibits selective downstream signaling. *J. Biol. Chem.* **278**, 50442-50448.

Menzel, N., Schneeberger, D. and Raabe, T. (2007). The Drosophila p21 activated kinase Mbt regulates the actin cytoskeleton and adherens junctions to control photoreceptor cell morphogenesis. *Mech. Dev.* **124**, 78-90.

Mira, J.-P., Benard, V., Groffen, J., Sanders, L. C. and Knaus, U. G. (2000). Endogenous, hyperactive Rac3 controls proliferation of breast cancer cells by a p21-activated kinase-dependent pathway. *Proc. Natl. Acad. Sci. USA* **97**, 185-189.

Nwariaku, F. E., Liu, Z., Zhu, X., Nahari, D., Ingle, C., Wu, R. F., Gu, Y., Sarosi, G. and Terada, L. S. (2004). NADPH oxidase mediates vascular endothelial cadherin phosphorylation and endothelial dysfunction. *Blood* **104**, 3214-3220.

Pirraglia, C., Jattani, R. and Myat, M. M. (2006). Rac function in epithelial tube morphogenesis. *Dev. Biol.* **290**, 435-446.

Quinlan, M. P. (1999). Rac regulates the stability of the adherens junctions and its components, thus affecting epithelial cell differentiation and transformation. *Oncogene* **18**, 6434-6442.

Rheinwald, J. G. (1989). Methods for clonal growth and serial cultivation of normal human epidermal keratinocytes and mesothelial cells. In *Cell Growth and Division. A Practical Approach* (ed. R. Baserga), pp. 81-94. Oxford: IRL Press.

Ridley, A. J., Comoglio, P. M. and Hall, A. (1995). Regulation of scatter factor/hepatocyte growth factor responses by Ras, Rac and Rho in MDCK cells. *Mol. Cell. Biol.* **15**, 1110-1122.

Sahai, E. and Marshall, C. J. (2002a). RHO-GTPases and cancer. *Nat. Rev. Cancer* **2**, 133-142.

Sahai, E. and Marshall, C. J. (2002b). ROCK and Dia have opposing effects on adherens junctions downstream of Rho. *Nat. Cell Biol.* **4**, 408-415.

Schnelzer, A., Prechtel, D., Knaus, U., Dehne, K., Gerhard, M., Graef, H., Harbeck, N., Schmitt, M. and Lengyel, E. (2000). Rac1 in human breast cancer: overexpression, mutation analysis,and characterization of a new isoform, Rac1b. *Oncogene* **19**, 3013-3020.

Sells, M. A., Knaus, U. G., Ambrose, D. M., Bokoch, G. M. and Chernoff, J. (1997). Human p21-activated kinase (Pak-1) regulates actin organization in mammalian cells. *Curr. Biol.* **7**, 202-210.

Shintani, Y., Wheelock, M. J. and Johnson, K. R. (2006). Phosphoinositide-3 Kinase-Rac1-c-Jun NH2-terminal kinase signaling mediates collagen I-induced cell scattering and up-regulation of N-cadherin expression in mouse mammary epithelial cells. *Mol. Biol. Cell* **17**, 2963-2975.

Singh, A., Karnoub, A. E., Palmby, T. R., Lengyel, E., Sondek, J. and Der, C. J. (2004). Rac1b, a tumor associated, constitutively active Rac1 splice variant, promotes cellular transformation. *Oncogene* **23**, 9369-9380.

Stappenbeck, T. S. and Gordon, J. I. (2000). Rac1 mutations produce aberrant epithelial differentiation in the developing and adult mouse small intestine. *Development* **127**, 2629-2642.

Takaishi, K., Sasaki, T., Kato, M., Yamoshi, W., Kuroda, S., Nakamura, T., Takeichi, M. and Takai, Y. (1994). Involvement of rho p21 small GTP-binding protein and its regulator in the HGF-induced cell motility. *Oncogene* **9**, 273-279.

Tang, Y., Chen, Z., Ambrose, D., Liu, J., Gibbs, J. B., Chernoff, J. and Field, J. (1997). Kinase-deficient PAK1 mutants inhibit Ras transformation of Rat-1 fibroblasts. *Mol. Cell. Biol.* **17**, 4454-4464.

Thiery, J. P. (2003). Epithelial-mesenchymal transitions in development and pathologies. *Cur. Opin. Cell Biol.* **15**, 740-746.

Wang, R.-A., Vadlamudi, R. K., Bagheri-Yarmand, R., Beuvink, I., Hynes, N. E. and Kumar, R. (2003). Essential functions of p21-activated kinase 1 in morphogenesis and differentiation of mammary glands. *J. Cell Biol.* **161**, 583-592.

Yagi, R., Waguri, S., Sumikawa, Y., Nada, S., Oneyama, C., Itami, S., Schmedt, C., Uchiyama, Y. and Okada, M. (2007). C-terminal Src kinase controls development and maintenance of mouse squamous epithelia. *EMBO J.* **26**, 1234-1244.

Zegers, M. M. P., Forget, M.-A., Chernoff, J., Mostov, K. E., ter Beest, M. B. A. and Hansen, S. H. (2003). Pak1 and PIX regulate contact inhibition during epithelial wound healing. *EMBO J.* **22**, 4155-4165.

Zhao, Z., Manser, E., Chen, X., Chong, C., Leung, T. and Lim, L. (1998). A conserved negative regulatory region in αPAK: inhibition of PAK kinases reveals their morphological roles downstream of Cdc42 and Rac1. *Mol. Cell. Biol.* **18**, 2153-2163.

Zhong, C., Kinch, M. S. and Burridge, K. (1997). Rho-stimulated contractily contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. *Mol. Biol. Cell* **8**, 2329-2344.